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Authors

Davis, Barbara
To, Vivian
Talbot, Prue

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Comparison of cytotoxicity of IQOS aerosols to smoke from Marlboro Red and 3R4F reference cigarettes

Barbara Davis (barbara.davis@email.ucr.edu) [a], Vivian To (vto002@ucr.edu) [a], and Prue Talbot (talbot@ucr.edu) [a]

[a] Department of Molecular, Cell and Systems Biology, University of California, Riverside, CA 92521, United States

Corresponding author: Prue Talbot

Address: Department of Cell, Molecular, and Systems Biology

University of California

Riverside, CA 92521

Email: talbot@ucr.edu

Telephone: 951-827-3768

FAX: 951-827-4286

ABSTRACT:

This study compared the cytotoxicity of IQOS aerosols to smoke from Marlboro Red (MR) and 3R4F reference cigarettes. Aerosol/smoke solutions were tested as the gas vapor phase (GVP), particulate phase (total particulate matter or TPM), or whole aerosol/smoke (WA), the latter being what smokers actually inhale. Cytotoxicities were evaluated using the LDH, MTT and neutral red uptake (NRU) assays in conjunction with eight different cell types, mainly from the respiratory system. Most test solutions did not compromise the plasma membranes of cells (LDH). However, mitochondrial activity (MTT) and dye uptake/lysosomal activity (NRU) were equally depressed by IOQS aerosols and cigarette smoke solutions at the high concentrations. Our NRU data with mouse 3T3 transformed fibroblasts were similar to those previously reported by the IQOS manufacturer and showed little cytotoxicity in the NRU assay. In both studies with 3T3 cells, the results were significantly different from 3RF4 cigarette smoke, suggesting reduced toxicity with IQOS. However, by expanding evaluations to a broader spectrum of cells that included respiratory system cells and by including higher concentrations of GVP, as well as WA, cytotoxicity equivalent to that of Marlboro Red and 3R4F cigarettes was frequently observed with IQOS aerosols in the MTT and NRU assays.

Keywords: IQOS, new tobacco products, heat-not-burn tobacco products, heated tobacco product, electronic nicotine delivery devices, cytotoxicity

INTRODUCTION

IQOS is a novel heat-not-burn cigarette or heated tobacco product released by Phillip Morris International (PMI) in 2014. Initially sold only to Japanese and Italian test markets, IQOS is now available in 41 countries and in Duty Free shops worldwide [1]. IQOS functions by heating a cast-leaf tobacco sheet, producing an aerosol without burning of the tobacco [2,3]. Although marketed as a harm reduction product, there is currently little published data on the health effects of IQOS aerosol. The manufacturer has written nine papers evaluating the IQOS system. Of these, only one focused on cytotoxicity [4]. In their study, a filter was used to separate the gas vapor phase (GVP), which passed through the filter, from total particulate matter (TPM). The GVP was captured in phosphate-buffered saline solution, while the TPM was captured on the filter and solubilized in dimethyl sulfoxide (DMSO) [4]. IQOS aerosols were tested for cytotoxicity on mouse embryonic fibroblasts (NIH/3T3) using the neutral red uptake assay (NRU). From these data, it was concluded that IQOS aerosols were less cytotoxic than 3R4F reference cigarettes.

The purpose of our study was to repeat the cytotoxicity tests done by Schaller et al. [4] and to broaden the screen to include eight cell types, three cytotoxicity assays, and Marlboro Red cigarettes in addition to 3R4F research cigarettes. Aerosol/smoke solutions were tested as GVP and TMP, emulating the

PMI method, and as whole aerosol/smoke (WA) collected in complete cell culture medium, which better models user exposure. IQOS aerosols were generated using two device cleanliness conditions, C1, in which the Holder was cleaned between each heatstick, and C20, in which the Holder was cleaned after the 20th heatstick, as described in the IQOS instruction manual. In a previous study, a lack of cleanliness in the IQOS Holder increased tobacco plug charring and polymer-film filter melting in the C20 samples [3].

One cell line from the mouse and seven cell types from humans were tested. NIH/3T3 cells are a hardy and fast-growing spontaneously transformed line of mouse embryonic fibroblasts, which were used in the PMI study [4]. In addition, we tested: (1) A549 cells isolated by others from an epithelial lung carcinoma; (2) BEAS-2B cells, an immortalized human bronchial epithelial cell line, often used for toxicity testing; (3) three primary human bronchial epithelial cell types (NHBE) from a child, an adult nonsmoker, and an adult smoker; (4) normal human lung fibroblasts (NHLF), which play a critical role in lung homeostasis, repair and remodeling and in previous studies have been highly sensitive to toxicant exposure [5,6]; and (5) H9 human embryonic stem cells (H9-hESC) which were used as an *in vitro* human embryo model.

Three assays were used to compare the cytotoxicity of IQOS to that of Marlboro Red (MR) and 3R4F research cigarettes. The assays were: (1) lactate dehydrogenase (LDH), which assesses cell viability/death through leakage of the plasma membrane; (2) (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT), which assesses metabolic activity via mitochondrial reductase

function; and (3) NRU, which assesses dye uptake by cells and sequestration in lysosomes. Using multiple cytotoxicity assays is important as treatments may not all affect the same endpoint.

MATERIALS & METHODS

Product Acquisition and Storage

IQOS Heat-not-burn kits (Phillip Morris Products S.A. (Switzerland) and cartons of IQOS Marlboro (blue box) heatsticks (Phillip Morris Brands Sàrl, (Italy) were purchased and stored as previous described [3]. Marlboro Red cigarettes (Philip Morris USA Inc., Richmond, VA) were purchased at Wal-Mart. 3R4F research cigarettes were purchased from the University of Kentucky.

Aerosol and Smoke Solution Production

IQOS aerosols were generated under two conditions, a per-use cleaning protocol (C1), in which the Holder was cleaned between each heatstick, and the manufacturer's recommended cleaning protocol (C20), in which the device was cleaned after the 20th heatstick. Smoke solutions were produced using MR and 3R4F reference cigarettes. Two types of aerosol/smoke solutions were produced, fractionated, which employed a 47 mm Single Stage Filter Assembly (Savillex, Eden Prairie, MN) outfitted with 47 mm Emfab membrane filter (Pall Life Sciences, Ann Arbor, MI), and complete medium, which did not utilize a filter. All aerosol/smoke solutions were made using the following smoking machine configuration which is shown in Figure 1: the mouthpiece filter end of the IQOS heatstick (inserted into the IQOS Holder) was inserted into one free end of a 3/8-inch T-Type connector (Thermo Scientific, Rochester, NY). The connector fit tightly and did not allow any air to be pulled into the smoking machine from outside of the heatstick. One end of the T-connector was used to block air flow allowing for the

activation of the puff, and the other end was either connected to the filter assembly (fractionated) or connected directly (complete medium) to two in-line glass absorption impingers, custom modified by Kimball Chase (Rockwood, TN). For fractionated method, the first impinger contained 50 mL of ice-cold Dulbecco's phosphate-buffered saline (DPBS) solution without Ca^{2+} and Mg^{2+} (Lonza, Walkersville, MD), while for WA, the impinger contained 50 mL of cell specific culture medium. For both, the second impinger contained ice-cold deionized water, both impingers were placed into an ice bath during the course of aerosol/smoke collection. The impingers were then connected to a Cole-Palmer Masterflex L/S peristatic pump (Vernon Hills, IL) equipped with a Cole-Palmer Masterflex L/S Easy-Load II Model 77200-52 high performance pump head and utilizing Masterflex Tygon E3603 (Tubing Size 36). This configuration allows for the application of the Health Canada standard (HCI) smoking protocol [7] which requires a 2 second puff that generates a total puff volume of 55 mL (27.5mL/sec), with an interpuff interval of 30 seconds.

Fractionated aerosols/smoke were composed of two parts, a gas vapor phase (GVP), which was immobilized in the DPBS of impinger one, and the total particulate matter (TPM) which was trapped onto the Emfab membrane filters and desorbed by solubilization in 50 mL of dimethyl sulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ). Filters were changed after every two heatsticks or cigarettes. For IQOS, a total of 11 Emfab membrane filters were used (12 puffs per heatstick for a total of 21 heatsticks) and for cigarette products a total of 13 (10 puffs per cigarette for a total of 25 cigarettes). All aerosol/smoke concentrations were

expressed as a percent of solution. All solutions were aliquoted into 450 μL volumes and placed in 0.5 mL locking lid microcentrifuge tubes (Fisher Scientific, Fair Lawn, NJ) to reduce headspace, and stored at -80ºC until needed. For each aerosol type, a total of 250 puffs were taken. Heatsticks were designed to have a maximum of 14 puffs but only 12 puffs were taken from each heatstick because this device automatically shuts off after 6 minutes of use and must be recharged.

MR cigarettes were smoked to a butt length of 35 mm, approximately 10 puffs/cigarette. The overall length of the 3R4F reference cigarettes was longer than MR by 5 mm. The filter portion of the reference cigarette was 8 mm longer than that of the MR and the tobacco filled portion was 3 mm shorter thus it was decided to mimic puff number when smoking the reference cigarette as opposed to butt length.

Cell Types and Cell Culture

NIH/3T3 mouse embryonic fibroblasts (NIH/3T3) (ATCC, Manassas, VA), A-549 human lung carcinoma cells (A-549) (ATCC, Manassas, VA), normal human lung fibroblasts (NHLF) (Lonza, Walkersville, MD), BEAS-2B (ATCC, Manassas, VA), all normal human bronchial epithelial cells (NHBE) (child, adult non-smoker, and adult smoker) (MatTek, Ashland, MA), and H9 human embryonic stem cells (H9-hESC) (WiCell, Madison, WI) were maintained and cultured as described in Online Supplemental Figure SP1.

Cytotoxicity Assays

For all cytotoxicity assays, lactate dehydrogenase (LDH), 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT), 3-amino-7dimethyl-2-methylphenazine hydrochloride (Neutral Red dye Uptake, NRU), cells were plated at cell-type specific densities (Online supplemental Figure SP1) and allowed to attach for 24 hrs. Cells were then treated with varying dilutions of 3% TPM, 30% GVP or 30% WA for 24 hrs (Online supplemental figure SP1). The concentration range was chosen to bracket doses that an IQOS user is likely to receive. After treatment, cells were subjected to either the LDH, MTT or NRU assays. For LDH, the threshold of cytotoxicity was determined as a 30% increase on the y-axis between the lowest and highest concentration. For MTT and NRU, the threshold of cytotoxicity was set at <70% of control, as determined by ISO 10993-5 [8]. Each experiment was performed three times. Statistical analyses of concentration-response data were performed using Minitab 18 software (State College, PA). Effects of treatment were determined using a two-way ANOVA followed by Fisher's post-hoc test with a Bonferroni correction (Tables 1-3 and SP 2-9). To rank cell type sensitivity, a one-way ANOVA with Fisher's post-hoc test was used (Table 4).

RESULTS

Overview of Testing

The cytotoxicities of IQOS (C1 and C20) and conventional cigarettes (MR and 3R4F) were evaluated for eight cell types using the MTT, NRU and LDH assays. Three types of aerosol fluid were compared for each IQOS/cigarette group. These were: (1) 3% TPM, (2) 3% GVP, and (3) 30% GVP. Five of the eight cell types (NIH/3T3, A549, NHLF, NHBE-smoker and nonsmoker) were also tested using 30% WA.

Figure 2 shows representative concentration-response graphs for 30% WA using NHBE-nonsmoker cells. In the LDH assay (Figure 2 A), none of the treatments produced cytotoxicity, indicating cell plasma membrane integrity was not compromised. In the MTT assay, the aerosols from conventional cigarettes were more cytotoxic than those from IQOS at the lower concentrations, but cytotoxicities were equivalent at 10 and 30% (Figure 2 B). In the NRU assay, cytotoxicities were similar for all four treatment groups (Figure 2 C). Concentration-response curves for all aerosol treatments and cell types are presented in supplemental data SP2-SP9 for each cytotoxicity assay.

Tables 1-3 report amalgamated data from the LDH, MTT and NRU assays, respectively. Data presented in these tables are the means of the percent of control for the highest tested concentration for each treatment and cell type. Statistical comparisons were done between each group and the 3R4F group, and treatments that were significantly different from the 3RF4 cigarettes are indicated by asterisks. Pale red boxes indicate that a treatment was cytotoxic (i.e., an IC_{70} or

>30% reduction from the untreated control was observed), and bright red boxes indicate IQOS values that were not significantly different from the 3R4F group. Green boxes indicate a lack of cytotoxicity. Pale blue boxes indicate cell type(s) that had statistical differences between C1 and C20 for 3% TPM and the bright blue box indicates cell type(s) that had statistical differences between C1 and C20 for 3% TPM and 30% WA. The next sections summarize the data for all treatments and cell types.

LDH Assay

All but four IQOS treatments and most conventional cigarette treatments were not cytotoxic with the LDH assay (Table 1). The most noteworthy results were: (1) for TPM, only five of 40 conventional cigarette treatments were cytotoxic (30% change from untreated control); (2) for 3% GVP, only two of 40 conventional cigarette treatments were cytotoxic; (3) for 30% GVP, 12 of 40 treatments were cytotoxic, and this included IQOS treatments for NHLF and BEAS-2B cells; and (4) for WA, cytotoxicities were only seen in six of 20 conventional cigarette treatments (NIH/3T3, A549 and NHLF). Two-way ANOVA results showed no statistical significance when comparing the effects of aerosol/smoke treatment and concentration on percent of control values. These data show that most treatments were not making cell plasma membranes leaky and killing cells. Additional assays were next used to determine if metabolism (MTT) and dye uptake and lysosomal integrity (NRU) were affected by treatments.

MTT Assay

Most 3% TPM test samples (33 of 40) were cytotoxic in the MTT assay (Table 2). Both conventional cigarettes were cytotoxic to all eight cell types, and IQOS C1 and C20 were cytotoxic to NIH/3T3, NHLF, BEAS-2B, and H9-hESC. The 3% TPM results for A549 (C1), NIH/3T3 (C1 and C20) and H9-hESC (C1 and C20) were not significantly different from 3R4F cigarettes (Table 2 red boxes), indicating that for these comparisons, IQOS and 3R4F cigarettes were equivalent in cytotoxicity.

The 3% GVP treatments were in general not cytotoxic. For conventional cigarette treatments, cytotoxicity was only observed for four cell types (NIH/3T3, NHLF, NHBE-child 3R4F and BEAS-2B MR), and IQOS treatments were not cytotoxic to any cell type. In contrast, 30% GVP was cytotoxic to almost all cells, the only exceptions being IQOS C1 and C20 for NIH/3T3 and A549 cells. Results with NHLF, NHBE-child (C20), and BEAS-2B were not significantly different from those with the 3RF4 group (Table 2 red boxes).

30% WA, which was tested with the five most relevant cell types, was generally cytotoxic. WA from both conventional cigarettes was cytotoxic to all cell types, and IQOS WA was cytotoxic to three of five cell types (NIH/3T3, NHLF, and NHBE-nonsmoker). The effect of WA C1 and C20 on NHBE-nonsmoker was not statistically different from 3R4F (Table 2 red boxes).

The MR and 3R4F groups were not statistically different from each other for any cell type or treatment group except NHLF exposed to 3% GVP, for which MR was significantly more cytotoxic than 3R4F ($p < 0.003125$). There were no

significant differences between C1 and C20 IQOS for any cell type or treatment group.

NRU Assay

For 3 % TPM, conventional cigarettes produced a cytotoxic effect for all eight cell types, while IQOS was cytotoxic to all cell types except NHBE-nonsmoker and NHBE-child. In some cases, either IQOS C1 (NIH 3T3, A549, NHBE-Smoker) or IQOS C20 (BEAS-2B) were cytotoxic. Among IQOS sensitive cells, A549 (C1) and H9-hESC (C1 and C2) were not statistically different from 3R4F treated cells (Table 3 red boxes).

3% GVP from IQOS was not cytotoxic to any cell type, while 3% GVP from cigarettes was cytotoxic only to NHLF. 30% GVP from conventional cigarettes was cytotoxic to all eight cell types, and IQOS was toxic to all but A549 cells. Of the seven cell types affected by IQOS, six were not significantly different from 3R4F (Table 3 red boxes). Only NIH 3T3 cells, the cell type tested previously by Schaller et al. [4], were significantly less affected by IQOS C1 and C20 than by the 3RF4 research cigarettes.

All 30% WA IQOS and conventional cigarette exposures were cytotoxic. For IQOS, the NBHE-smoker and NBHE-nonsmoker groups were not statistically different from the 3R4F group (Table 3 red boxes).

MR was significantly more cytotoxic than 3RF4 for the NHLF. Significant differences between the IQOS C1 and IQOS C20 groups were observed for TPM treatments of A549, NHLF, and NHBE-smoker (pale blue boxes in Table 3), and NIH/

3T3 had significant differences for TPM and WA treatments (bright blue box in Table 3).

Cell Type Sensitivity Hierarchy

Using one-way ANOVA analysis of 30% GVP data, cell types were ranked for overall sensitivity to aerosol/smoke exposure. Table 4 presents the means of three experiments for each cell type in the MTT and LDH assays and their sensitivity to treatment (grouping) based on the ANOVA analysis. In grouping, cells with the different letters were significantly different from each other. Cells were ranked in increasing sensitivity from A through C/D. For MTT data, A549 and NIH/3T3 were the least sensitive, the three NHBE were in the midrange, and NHLF, BEAS-2B and H9-hESC were the most sensitive to treatment (Table 4 MTT).

For NRU data, A549 and NHBE-child cells were the least affected by exposure, NHBE-nonsmoker, NHBE-smoker, and NIH/3T3 were mid-range, and NHLF, BEAS-2B and H9-hESC were the most sensitive (Table 4 NRU).

To determine an overall hierarchy of sensitivity, the mean values from MTT and NRU were averaged showing that A549 and NIH/3T3 were the least sensitive, the three NHBE were in the midrange, and NHLF, BEAS-2B and H9-hESC were the most sensitive (Table 4 Averaged).

DISCUSSION

The cytotoxicities of conventional cigarette smoke and IQOS aerosols were compared in a comprehensive screen using eight cell types, three endpoint assays, and various components of smoke/aerosols. NIH/3T3 cells and the NRU assay were chosen to allow direct comparison to a prior study on IQOS and 3R4F cigarettes [4]. Additional cells from respiratory tissue were included as relevant models for inhalation toxicology, and H9-hESC were studied to determine how embryos and hence prenatal development may be affected by IQOS. Fractions of smoke/aerosol (TMP and GVP) were compared, as was done previously [4], and whole aerosol and smoke, which is what IQOS users actually inhale, were also studied. Our five most significant observations were: (1) IQOS exposure did not lead to cell death (LDH) in most trials but did adversely affect critical cellular functions (MTT & NRU); (2) mouse 3T3 fibroblasts were not significantly affected by IQOS aerosol in the NRU assay, as reported previously by the manufacturer [4]; (3) for some cell types (bright red boxes in Tables 2 and 3), IQOS aerosol and 3R4F smoke were equally cytotoxic for comparisons made at the high concentrations; (4) cell types varied in their sensitivity to IQOS aerosol and cigarette smoke with cells from human embryos and the respiratory system usually being more sensitive than NIH/3T3, which were used in a prior study [4], and cancer cells (A549); (5) results for IQOS C1 and C20 were similar for the MTT assay, but NRU analysis showed differences between the treatments in A549 (C1 > C20), NHBE-smoker (C1 > C20), and NHLF (C1 < C20) for TMP and in NIH/3T3 for both TPM (C1 > C20) and WA (C1 < C20);

and (6) in all but one case (NHLF at 3% GVP), MR had equivalent cytotoxicities to 3R4F cigarettes.

LDH analysis showed that cells were generally not killed by IQOS, MR or 3R4F treatments. Notable exceptions were the NHLF and BEAS-2B cells, which were affected by all 30% GVP treatments. MTT and NRU data showed that IQOS C1 and C20 did have adverse effects on cell metabolism and dye uptake. These data agree with another study that investigated the effects of IQOS aerosols using an air-liquid interface (ALI) system and found that exposure did not lead to cell death but rather adversely affected metabolic activity [9]. Our MTT data are also in agreement with a recent study showing that IQOS had similar effects to cigarette smoke when tested with BEAS2-B cells, although these authors found that IQOS had a stronger effect in the LDH assay than we observed [23]. Comparison of our MTT and NRU data showed that both the 3% TPM and 30% GVP treatments were cytotoxic and that the response to GVP was concentration dependent. For WA, both IQOS and conventional cigarettes were cytotoxic to all five cell types with which they were tested. IQOS Holder cleanliness did not significantly affect the outcome for the MTT assay but did sometimes produce a significant effect in the NRU assay, with C20 usually being more cytotoxic than C1. In general, MR and R3F4 smoke was equivalent in both the MTT and NRU assays.

In a prior study on IQOS cytotoxicity using NIH/3T3 cells [4], the equivalent of IQOS C20 produced very little effect using the NRU, in agreement with our observations (e.g. 85% for 3% TPM and 88% for 3% GVP), and these effects in both studies were significantly different from 3RF4 cigarette smoke, suggesting reduced

toxicity with IQOS. However, by expanding evaluations to a broader spectrum of cells that included six types of respiratory cells and by including higher concentrations of GVP as well as WA, it was evident that cytotoxicity was frequently observed with IQOS aerosols. One of the most important observations in our study is the finding that in a number of instances, there was no significant difference in toxicity between IQOS and 3R4F treatments.

A hierarchy of cell sensitivity was created for the MTT and NRU assays and averages of these assays based on the 30% GVP data (Table 4). The most sensitive respiratory cells were NHLF, which play a vital role in maintaining lung health by producing the extracellular matrix, which is essential for support and normal lung function [10]. H9-hESC were also in the most sensitive group suggesting that IQOS may not be an appropriate product for pregnant women. All three primary, untransformed NHBE cell types (smoker, nonsmoker, and child) were in the midrange of sensitivity. Within the bronchial epithelial group, the NHBE cells from the smoker were always more sensitive than those from the adult non-smoker or child. While this hierarchy is based on cells from only one individual per group, these data suggest that the respiratory epithelium from a smoker is less able to tolerate IQOS aerosol exposure than similar cells from non-smokers and children. NIH/3T3 (mouse embryonic fibroblast) and A549 cells (lung carcinoma) were the least sensitive to IQOS treatment, indicating they may not be the best cell choice for testing tobacco products that are inhaled. These data reaffirm that different cell types should be evaluated in cytotoxicity testing and show that cells from the

human respiratory system are more sensitive to IQOS treatment than mouse 3T3 cells, which were used in a prior study [4].

Although IQOS aerosol did not kill cells, it did have adverse effects in the MTT and NRU assays. These assays examine different endpoints and provide information on how IQOS aerosol affects cellular functions. A decline in mitochondrial reductase activity, as measured by the MTT assay, can lead to metabolic dysfunction, causing increased ROS production and oxidative damage [11-13]. This dysfunction can adversely affect redox signaling, which regulates cell death and survival pathways [12,14]. Decreases in reductase activity also lowers ATP production by oxidative phosphorylation [14-17], leading to compromised cell health. Dysregulation of succinate dehydrogenase, a key mitochondrial reductase and tumor suppressor, can lead to promotion of malignant cancers [18-20].

NRU data demonstrated that IQOS adversely effected dye uptake through the plasma membrane and/or maintenance of an acidic lysosomal pH. Proper pH gradients across lysosomal membranes require ATP production [21], thus our results with the NRU assay would be consistent with the observed decline in mitochondrial reductase (MTT assay). While the MTT and NRU assays gave similar results in our study, cytotoxicity was observed somewhat more frequently with the NRU assay (18 out of 32 trials vs 14 out of 32 trials for MTT), suggesting that NRU may be somewhat more sensitive than the MTT assay and may be a better choice if only one assay is used. Lysosomes play a critical role in cellular homeostasis by recycling of macromolecules, but when damaged, hydrolases leak out leading to lysosomal cell death (LCD) [22]. While we did not observe cell death in most trials

(LDH), the NRU data suggest that death would have occurred had incubations been longer.

The equivalent cytotoxicity observed with IQOS aerosol and 3R4F research cigarette smoke and the sensitivity of human bronchial epithelial cells and lung fibroblasts to IQOS aerosols is a concern. While our data cannot be directly extrapolated to human health, they clearly show a need for additional studies on IQOS products.

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COMPETING INTERESTS

None

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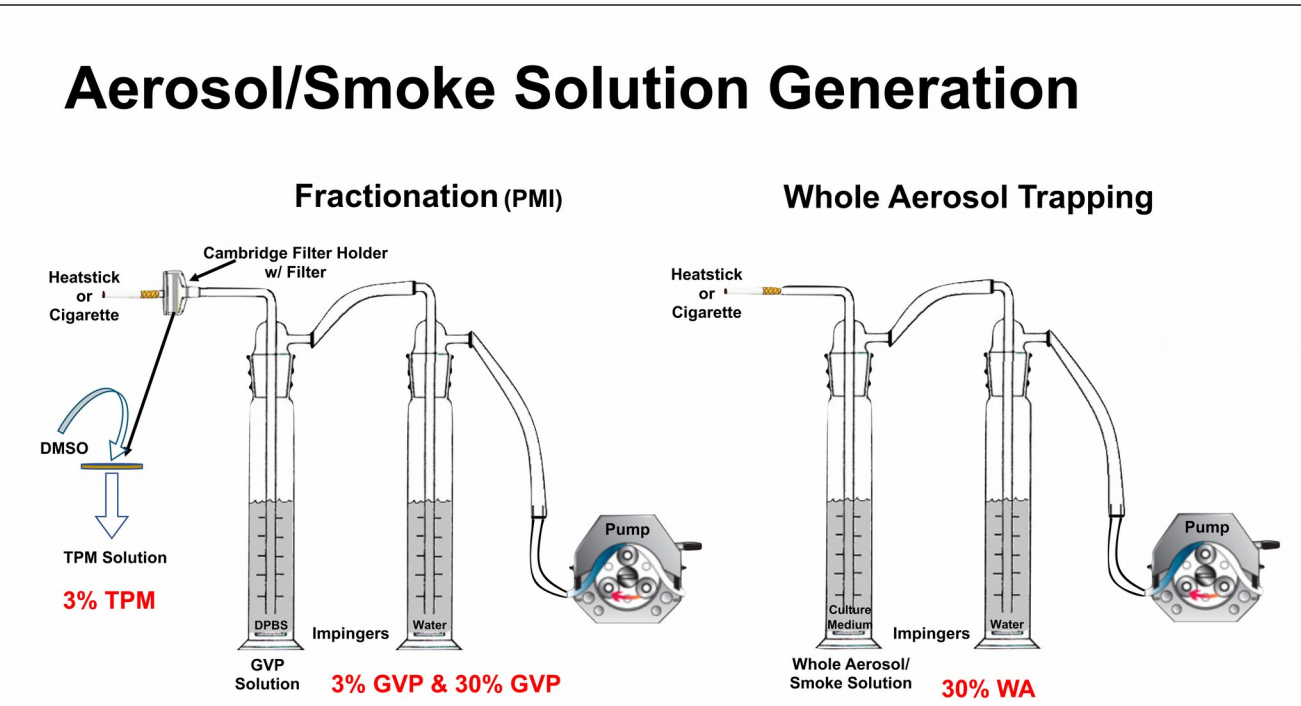


Figure 1: Diagram showing the configuration of the smoking machine used to make TPM, GVP, and WA.

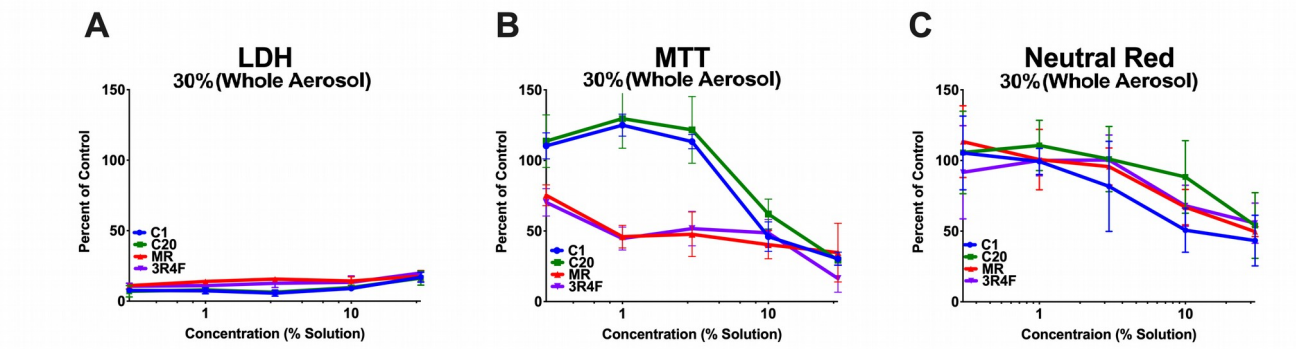


Figure 2. Representative concentration-response graphs for NHBE-nonsmoker treated with 30% WA. (A) LDH, (B) MTT, and (C) NRU assays. For all graphs x-axis = concentration in % solution, y-axis = percent of experimental control. Blue = C1, green = C20, red = Marlboro Red, purple = 3R4F. Each line represents the mean ± SD of three independent experiments.

Table 1: LDH Assay[1]

		LDH			
		3% TPM	3% GVP	30% GVP	30% WA
NIH/3T3	C1	44±3	48±2	50±4	12±2
	C20	41±1	42±3	53±4	11±2
	MR	41±0	45±1	64±4	46±3
	3R4F	39±1	44±1	65±4	31±3
A549	C1	17±3	18±2	17±2	13±1
	C20	17±1	17±1	19±1	14±0
	MR	16±1	16±1	15±1	27±1
	3R4F	17±1	16±2	16±1	28±2
NHLF	C1	14±2	21±2	43±1	9±1
	C20	16±1	20±2	44±3	10±1
	MR	28±1	36±2	37±3	57±4
	3R4F	19±5	26±4	32±2	60±5
NHBE-Smoker	C1	16±4	16±3	17±2	16±5
	C20	10±1	18±1	21±2	13±1
	MR	31±1	17±2	31±1	24±6
	3R4F	27±4	19±2	31±3	19±4
NHBE-Nonsmoker	C1	9±1	18±5	16±1	17±3
	C20	11±2	15±1	20±2	16±5
	MR	23±3	12±0	23±4	18±4
	3R4F	27±1	19±1	22±2	20±2
NHBE-Child	C1	8±1	16±1	20±3	n/a
	C20	11±1	12±1	23±1	n/a
	MR	25±1	14±1	33±4	n/a
	3R4F	21±1	15±1	31±6	n/a
BEAS-2B	C1	2±1	2±1	35±4	n/a
	C20	2±1	2±1	45±3	n/a
	MR	8±1	15±1	31±4	n/a
	3R4F	6±1	3±1	37±3	n/a
H9-hESC	C1	26±3	23±1	20±2	n/a
	C20	27±4	25±1	23±4	n/a
	MR	29±1	26±1	28±1	n/a
	3R4F	33±5	24±1	46±3	n/a

[1]LDH cytotoxicity data for IQOS and conventional cigarettes. Columns show the four types of aerosol/smoke solution that were tested. Rows show the cell types and product source of the aerosol/smoke solutions. Data are the means ± standard deviations of three experiments at the highest concentration tested for each type of aerosol/smoke solution. C1, C20, and MR red values were statistically compared to 3R4F using two-way ANOVAs, TPM = total particulate matter of fractionated aerosol/smoke; GVP = gas vapor phase of fractionated aerosol/ smoke; WA = whole aerosol/smoke captured in culture medium. C1 = IQOS cleaned after each use; C20 = IQOS cleaned after 20 uses. MR = Marlboro Red cigarettes; 3R4F = reference cigarette. Pale red boxes = toxicity values < 70% of control, green boxes = toxicity values ≥ 70%, bright red boxes = IQOS and R3R4F means were not significantly different.

Table 2 MTT Assay [1]

		MTT			
		3% TPM	3% GVP	30% GVP	30% WA
NIH/3T3	C1	61±9	75±12	87±5*	41±6*
	C20	51±20	91±8*	104±14*	43±6*
	MR	30±2	66±5	44±4	18±2
	3R4F	49±4	68±6	35±5	8±3
A549	C1	59±24	97±4	88±4*	75±6*
	C20	70±19*	95±3	93±3*	73±3*
	MR	46±7	87±4	49±1	11±1
	3R4F	41±2	100±2	46±4	9±1
NHLF	C1	38±9*	84±16*	7±2	62±4*
	C20	47±3*	93±10*	7±2	67±5*
	MR	18±2	23±4*	5±3	5±1
	3R4F	19±4	51±6	11±3	5±1
NHBE-Smoker	C1	77±5*	108±13	53±18*	83±4*
	C20	81±14*	102±3	41±17*	96±6*
	MR	35±6	94±3	11±1	29±2
	3R4F	33±5	100±4	8±1	25±1
NHBE-Nonsmoker	C1	77±3*	107±5	54±22*	30±5
	C20	81±12*	117±18	47±22*	29±2
	MR	35±7	96±10	15±4	35±21
	3R4F	33±5	104±22	11±1	16±10
NHBE-Child	C1	90±35*	80±19	50±14*	n/a
	C20	86±6*	94±17	44±9	n/a
	MR	50±15	87±15	16±1	n/a
	3R4F	44±22	59±13	12±2	n/a
BEAS-2B	C1	40±6*	89±6*	12±5	n/a
	C20	52±6*	88±21	9±10	n/a

			*		
	MR	7±1	24±7	2±0	n/a
	3R4F	6±1	70±14	2±0	n/a
H9-hESC	C1	17±5	86±8	11±3*	n/a
	C20	11±5	102±11	13±3*	n/a
	MR	17±10	78±8	3±2	n/a
	3R4F	12±5	91±20	3±1	n/a

[1]MTT cytotoxicity data for IQOS and conventional cigarettes. Columns show the four types of aerosol/smoke solution that were tested. Rows show the cell types and product source of the aerosol/smoke solutions. Data are the means of three experiments at the highest concentration tested for each type of aerosol/smoke solution. C1, C20, and MR red values were statistically compared to 3R4F using two-way ANOVA, * = adjusted $p < 0.003125$. TPM = total particulate matter of fractionated aerosol/smoke; GVP = gas vapor phase of fractionated aerosol/smoke; WA = whole aerosol/smoke captured in culture medium. C1 = IQOS cleaned after each use; C20 = IQOS cleaned after 20 uses. MR = Marlboro Red cigarettes; 3R4F = reference cigarette. Pale red boxes = toxicity values < 70% of control (considered cytotoxic), green boxes = toxicity values ≥ 70% (not cytotoxic), bright red boxes = IQOS and R3R4F means were not significantly different. * indicates the result was significantly different than 3R4F.

Table 3

NRU

		Neutral Red Assay[1]			
		3% TPM	3% GVP	30% GVP	30% WA
NIH/3T3	C1	63±8*	80±6	52±4*	58±1*
	C20	85±4*	88±5*	59±5*	39±1*
	MR	24±3	83±7	11±2	15±2
	3R4F	30±4	75±6	13±1	10±4
A549	C1	50±5	106±4	89±9*	63±6*
	C20	79±18	105±21	86±5*	65±4*
	MR	46±5	93±5	43±4	23±3
	3R4F	60±18	95±2	39±7	22±2
NHLF	C1	62±9*	88±3*	20±3	42±2*
	C20	47±6*	91±2*	22±7	46±3*
	MR	17±3	34±4*	15±1	7±1
	3R4F	28±8	59±4	17±2	7±1
NHBE-Smoker	C1	68±15*	81±15	49±16	38±7
	C20	96±1*	80±7	48±7	32±5
	MR	40±14	95±5	36±8	37±4
	3R4F	35±2	78±10	45±8	39±1
NHBE-Nonsmoker	C1	109±9*	95±14	66±6	43±4
	C20	77±8*	91±15	43±6	54±4
	MR	40±3	88±4	47±27	50±4
	3R4F	33±5	77±5	46±8	56±2
NHBE-Child	C1	83±14	98±9	62±7	n/a
	C20	70±12	100±19	69±13	n/a
	MR	47±3	79±6	54±7	n/a
	3R4F	47±8	82±5	69±10	n/a
BEAS-2B	C1	72±2*	78±15	17±4	n/a
	C20	59±1*	83±5	16±4	n/a
	MR	45±13	73±14	19±7	n/a
	3R4F	35±8	74±4	13±2	n/a
H9-hESC	C1	24±9	96±15	22±9	n/a
	C20	27±14	109±22	31±8	n/a
	MR	23±11	90±24	12±6	n/a
	3R4F	19±2	105±15	22±4	n/a

[1]NRU cytotoxicity data for IQOS and conventional cigarettes. Columns show the four types of aerosol/smoke solution that were tested. Rows show the cell types and product source of the aerosol/smoke solutions. Data shown are the means of three experiments at the highest concentration tested for each type of aerosol/smoke solution. C1, C20, and MR red values were statistically compared to 3R4F using a two-way ANOVA, * = adjusted $p < 0.003125$. TPM = total particulate matter of fractionated aerosol/smoke; GVP = gas vapor phase of fractionated aerosol/ smoke; WA = whole aerosol/smoke captured in culture medium. C1 = IQOS cleaned after each use; C20 = IQOS cleaned after 20 uses. MR = Marlboro Red cigarettes; 3R4F = reference cigarette. Pale red boxes = toxicity values < 70% of control (considered cytotoxic), green boxes = toxicity values ≥ 70% of the control (non-cytotoxic), bright red boxes = IQOS and R3R4F means are not significantly different. Bright blue box = cell type(s) that had statistical differences between C1 and C20 for 3% TPM and 30% WA. Pale blue boxes = cell type(s) that had statistical differences between C1 and C20 for 3% TPM. * indicates the result was significantly different than 3R4F.

Table 4: Cell Type Sensitivity Hierarchy[1]

	MTT			**NRU**			**Averaged[1]**	
Cell Type	Mean (SD)	Grouping[2]	Cell Type	Mean (SD)	Grouping[2]	Cell Type	Mean (SD)	Grouping[2]
A549	68.83 (24.94)	A	A549	64.17 (26.92)	A	A549	66.50 (24.16)	A
NIH/ 3T3	67.67 (33.27)	A	NHBE-C	63.25 (7.14)	A	NIH/ 3T3	50.84 (32.77)	A, B
NHBE-NS	30.42 (21.9)	B	NHBE-NS	56.08 (10.47)	A, B	NHBE-C	47.38 (22.19)	B, C
NHBE-C	31.5 (19.28)	B	NHBE-S	44.5 (5.92)	B, C	NHBE-NS	43.25 (18.79)	C
NHBE-S	28.42 (22.23)	B	NIH/ 3T3	34 (25.29)	C	NHBE-S	36.46 (17.39)	C
NHLF	7.33 (2.52)	C	H9	21.17 (7.76)	D	H9	14.42 (9.78)	D
H9	7.67 (5.26)	C	NHLF	18.5 (3.11)	D	NHLF	12.92 (6.44)	D
B2B	6.25 (5.06)	C	B2B	16.33 (2.5)	D	B2B	11.29 (6.50)	D

[1]Cell type treatment sensitivity hierarchy. Using one-way ANOVA with Fisher post-hoc test, cells were ranked based on mean percent of control values of three experiments vs. cell type. The grouping column shows the results of the ANOVA. Means that do not share a letter (Groupings) are significantly different. Tables were arranged from least sensitive to most sensitive for MTT and NRU data and the average of MTT and NRU means.

Supplementary Material and Methods and Figures

SP1: Materials and Methods:

Cell Culture

NIH/3T3 mouse embryonic fibroblasts (ATCC, Manassas, VA) on Nunc™ Cell Culture Treated EasYFlasks™ (T-25 flasks) (Roskilde, Denmark) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% bovine calf serum (ATCC, Manassas, VA) with twice weekly medium changes until cells were ≤ 80% confluent. To subculture or prepare for experiments, cells were rinsed in 0.25% trypsin-EDTA (1x) (Gibco, Grand Island, NY) then detached using 0.25% trypsin-EDTA (1x) for 5 min at 37ºC. Trypsin was neutralized by adding complete growth medium in double the volume of trypsin, and cell suspensions were aliquoted into appropriate culture vessels. For subculture, cells were plated at 4 x 10^3 cells/cm^2 (1 x 10^5 cells/T-25 flask); for cytotoxicity experiments, cells were plated at 2.5 x 10^3 cells/well (of a 96-well plate, Falcon, Corning, Durham, NC).

H9-hESC (WiCell, Madison, WI), on 6-well tissue culture treated plates (Falcon, Corning, Durham, NC) coated in Matrigel Matrix (Corning, Bedford, MA), were maintained in mTeSR™1 medium (Stem Cell Technologies, Vancouver, BC, Canada) with daily medium changes until cells were when 60–80% confluent. For subculture and experimental preparation of cells, wells were washed in DPBS, colonies were then detached using ReLeSR™ enzyme-free passaging reagent (Stem Cell Technologies, Vancouver, BC, Canada) for 5 min at room temperature, mTeSR™1 medium was added and the plate gently tapped to release colonies.

Cells were transferred to a 15 mL conical tube (Falcon, Corning, Durham, NC), the colonies were broken up by pipetting gently, cell suspensions were aliquoted into appropriate culture vessels. For subculture, cells were plated at 350 colonies/well (6-well plate); for cytotoxicity experiments, cells were plated at 150 colonies/well (96-well plate).

NHLF (Lonza, Walkersville, MD) on Nunc™ Cell Culture Treated EasYFlasks™ (T-25 flasks) were maintained in FGM™-2 Growth Medium (Fibroblast basal medium supplemented with FGM™-2 SingleQuots™) (Lonza, Walkersville, MD) with medium changes occurring every other day until cells reached a confluence of 70-80%. For subculture/experimental preparation, cells were washed in HEPES buffered saline solution, then detached using 0.25 mg/mL trypsin/EDTA solution for 2 min at 37ºC. Trypsin was neutralized with TNS (Trypsin neutralizing solution) (Lonza, Walkersville, MD), and cells were transferred to a 15 mL conical tube, rinse flask with HEPES and add to cell solution, centrifuge cells at 220xg for 5 min, discard supernatant, resuspend cells in culture medium, and aliquot cell suspensions into appropriate culture vessels. For subculture, cells were seeded at $2.5x10^3$ cells/cm^2 ($6.25x10^4$ cells/ T-25 flask); for cytotoxicity experiments cells were plated at $5x10^3$ cells/well (96-well plate).

A549 human lung carcinoma cells (ATCC, Manassas, VA) on Nunc™ Cell Culture Treated EasYFlasks™ (T-25 flasks) were maintained in F-12K medium supplemented with 10% fetal bovine serum (ATCC, Manassas, VA). Medium was changed every other day until cells reached a confluence ≤80%. Subculture/ experimental preparation, rinse cells in 0.25% Trypsin-EDTA (1x), detach using

0.25% Trypsin-EDTA (1x) for 2 min at 37ºC, neutralize trypsin by adding fetal bovine serum (Sigma Aldrich, St. Louis, MO) in double the volume of trypsin, transfer cell solution to a 15 mL conical tube, centrifuge cells at 220xg for 5 min, discard supernatant, resuspend cells in culture medium, and aliquot cell suspensions into appropriate culture vessels. For subculture, cells were seeded at $2.5x10^3$ cells/cm^2 ($6.25x10^4$ cells/T-25 flask); for cytotoxicity experiments cells were plated at $2.5x10^3$ cells/well (96-well plate).

BEAS-2B (ATCC, Manassas, VA) on Nunc™ Cell Culture Treated EasYFlasks™ (T-25 flasks) coated with 0.1 mg/mL Fibronectin, 0.03 mg/mL bovine collagen type I, and 0.01 mg/mL bovine serum albumin (Sigma Aldrich, St. Louis, MO) in BEBM™ basal medium (Lonza, Walkersville, MD) were maintained in BEGM™ (Bronchial epithelial basal medium supplemented with BEGM™ BulletKit™) (Lonza, Walkersville, MD) with every other day medium changes until cells reach a confluence of ≤80%. Subculture/ experimental preparation, cells are detached using 0.25% Trypsin-EDTA (1x) supplemented with 0.5% polyvinylpyrrolidone (Sigma Aldrich, St. Louis, MO) for 2 min at 37ºC, neutralize trypsin by adding complete growth medium in double the volume of trypsin, transfer cell solution to a 15 mL conical tube, centrifuge cells at 125xg for 5 min, discard supernatant, resuspend cells in culture medium, and aliquot cell suspensions into appropriate culture vessels. For subculture, cells were seeded at $2.25x10^3$ cells/cm^2 ($5.625x10^4$ cells/T-25 flask); for cytotoxicity experiments cells were plated at $3x10^3$ cells/well (96-well plate).

For all NHBE cell types (child, adult non-smoker, and adult smoker) on Nunc™ Cell Culture Treated EasYFlasks™ (T-25 flasks) were maintained NHBE-GM, growth medium, (NHBE-BM, basal medium, supplemented with NHBE-GS, growth serum, and NHBE-HCS, hydrocortisone) with medium changes occurring every other day until cells reached a confluence ≤80%. Subculture/ experimental preparation, rinse cells in DPBS, detach using 0.25% Trypsin-EDTA (1x) for 10 min at 37ºC, neutralize trypsin by adding STI (soybean trypsin inhibitor solution, 250 μg/mL), transfer cell solution to a 15 mL conical tube, rinse flask with DPBS and add to contents of conical vial, centrifuge cells at 150xg for 10 min, discard supernatant, resuspend cells in culture medium, and aliquot cell suspensions into appropriate culture vessels. For subculture, cells were seeded at $3.3x10^3$ cells/cm^2 ($8.25x10^4$ cells/T-25 flask); for cytotoxicity experiments cells were plated at $5x10^3$ cells/well (96-well plate).

All cultures were maintained at 37ºC with 5% CO2, and 95% relative humidity. For sub-culturing and experiments, cell counts were performed using a Nikon Eclipse TS100 Inverted Microscope (Tokyo, Japan) and a hemocytometer (Hausser Scientific, Horsham, PA), with the exception of the H9-hESC. For H9, colony counts were performed using a 96-plate following the protocol as described in the mTeSR1 product manual.

Cytotoxicity Assays

MTT

Thiazolyl Blue Tetrazolium Blue (MTT) (Sigma Aldrich, St. Louis, MO) solution was prepared at a concentration of 5 mg/mL in DPBS with Ca^{2+} and Mg^{2+} (Lonza, Walkersville, MD). After 24-hour of treatment, 20 µL of MTT was added to each well of cells and blank wells and incubated at 37ºC, 5% CO2, and 95% relative humidity for 2 hours. After incubation, MTT solution was removed and 100 µL of DMSO was added to each well. Solutions were gently mixed by pipetting. Plates were read using an Epoch microplate spectrophotometer (BioTek, Winooski, VT) at λ=570nm.

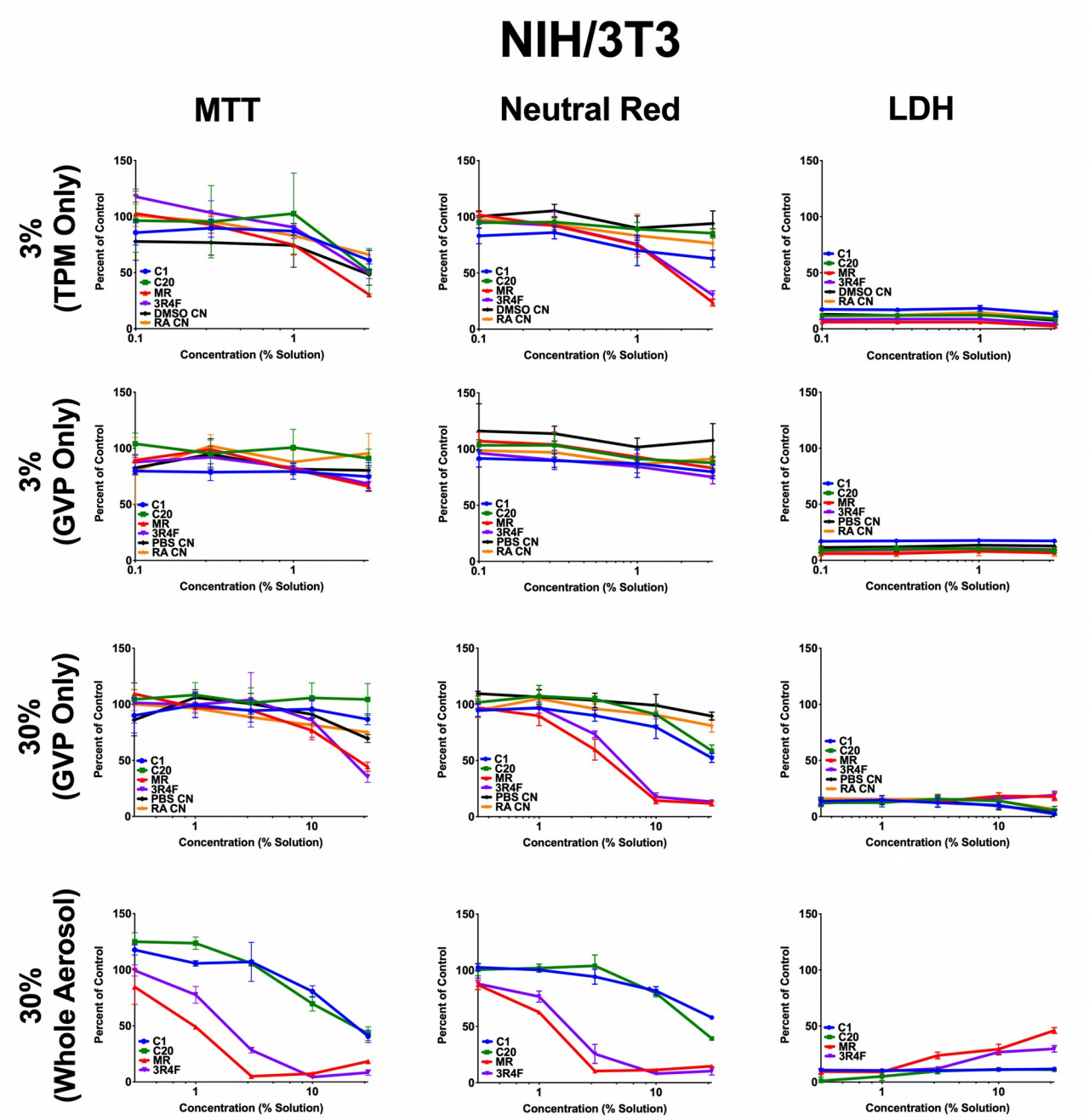
Neutral Red

Neutral Red biological stain (Acros Organics, NJ) was diluted in DPBS without Ca^{2+} and Mg^{2+} to a stock concentration of 4mg/mL. The stock was light shielded and stored at room temperature until needed. Neutral Red stock was further diluted to a working concentration of 40 µg/mL in cell type specific culture medium. Solutions were filtered and incubated with at 37°C overnight prior to addition to cells (neutral red solutions were prepared at the time of treatment). After 24-hour treatment, medium was removed from all wells and 150 µL of incubated/filtered, Neutral Red solution was added to each control treatment, and blank well. Plates were incubated at 37ºC, 5% CO2, and 95% relative humidity for 2 hours. During incubation a lysis solution of 50% ethanol, 49% $_dH_2O$ and 1% acetic acid (Fisher Scientific, Fair Lawn, NJ) was prepared. After incubation Neutral Red solution was removed, each well was rinsed with 100 µL DPBS without Ca^{2+} and Mg^{2+}, and 150 µL of lysis solution was added to each well and gently pipetted, ensuring no

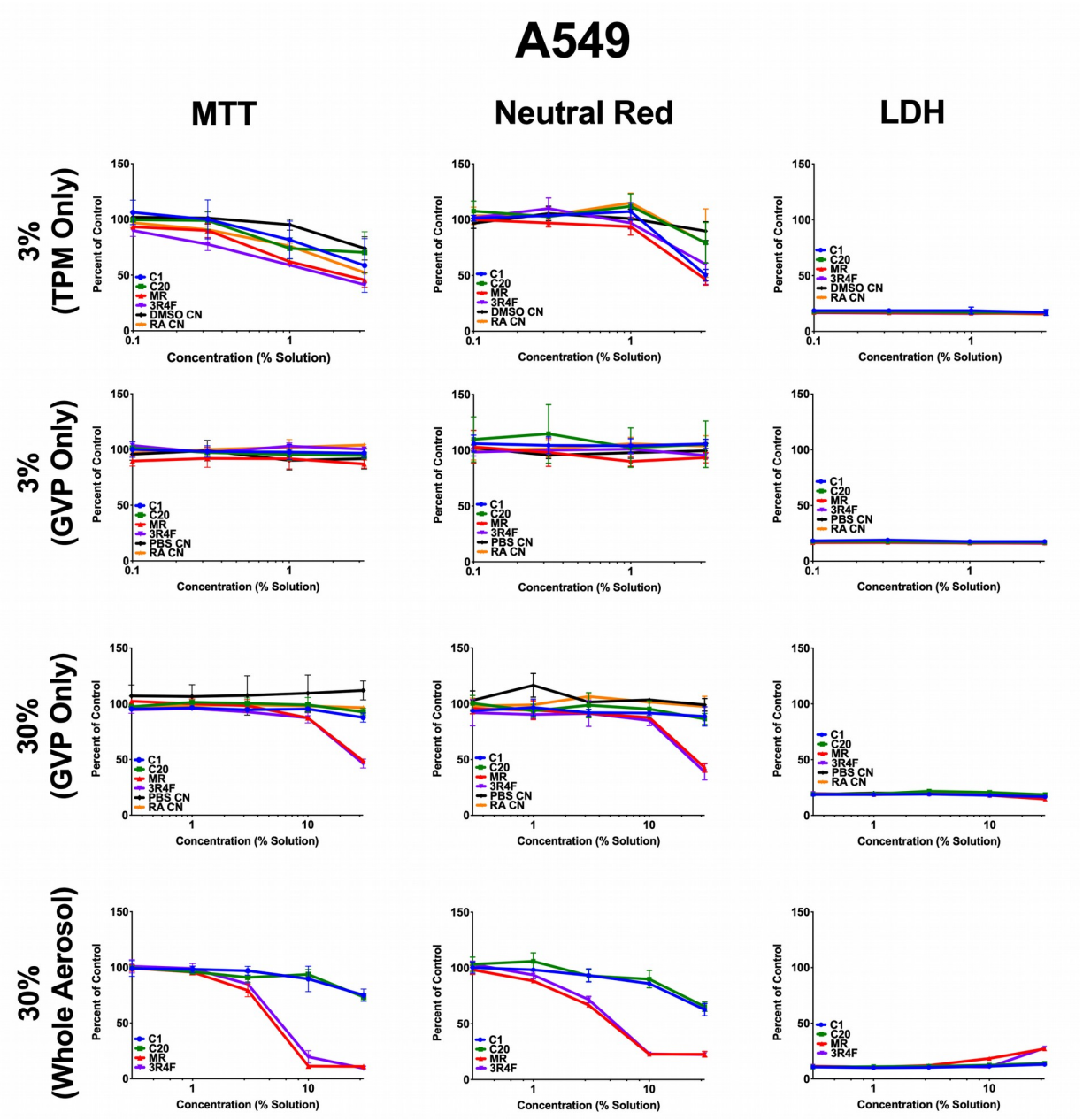
bubbles were present. Plates were read using an Epoch microplate spectrophotometer at λ=540nm.

LDH

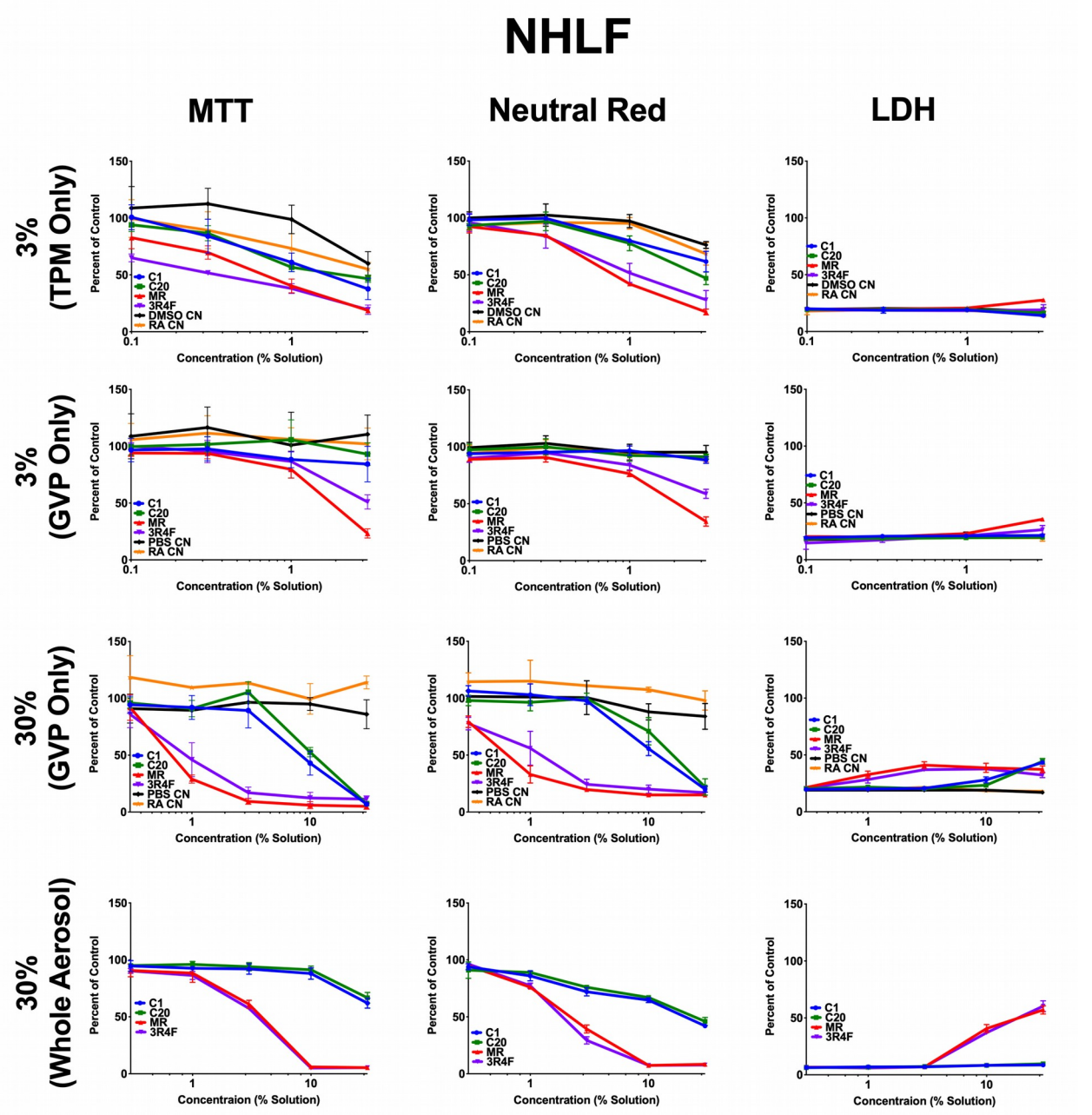
Pierce LHD Cytotoxicity Assay Kit (Pierce Biotechnology, Rockford, IL) was used to determine LDH values. All reagents were prepared, experiments were performed, and values were calculated according to the manufacturer’s protocol.



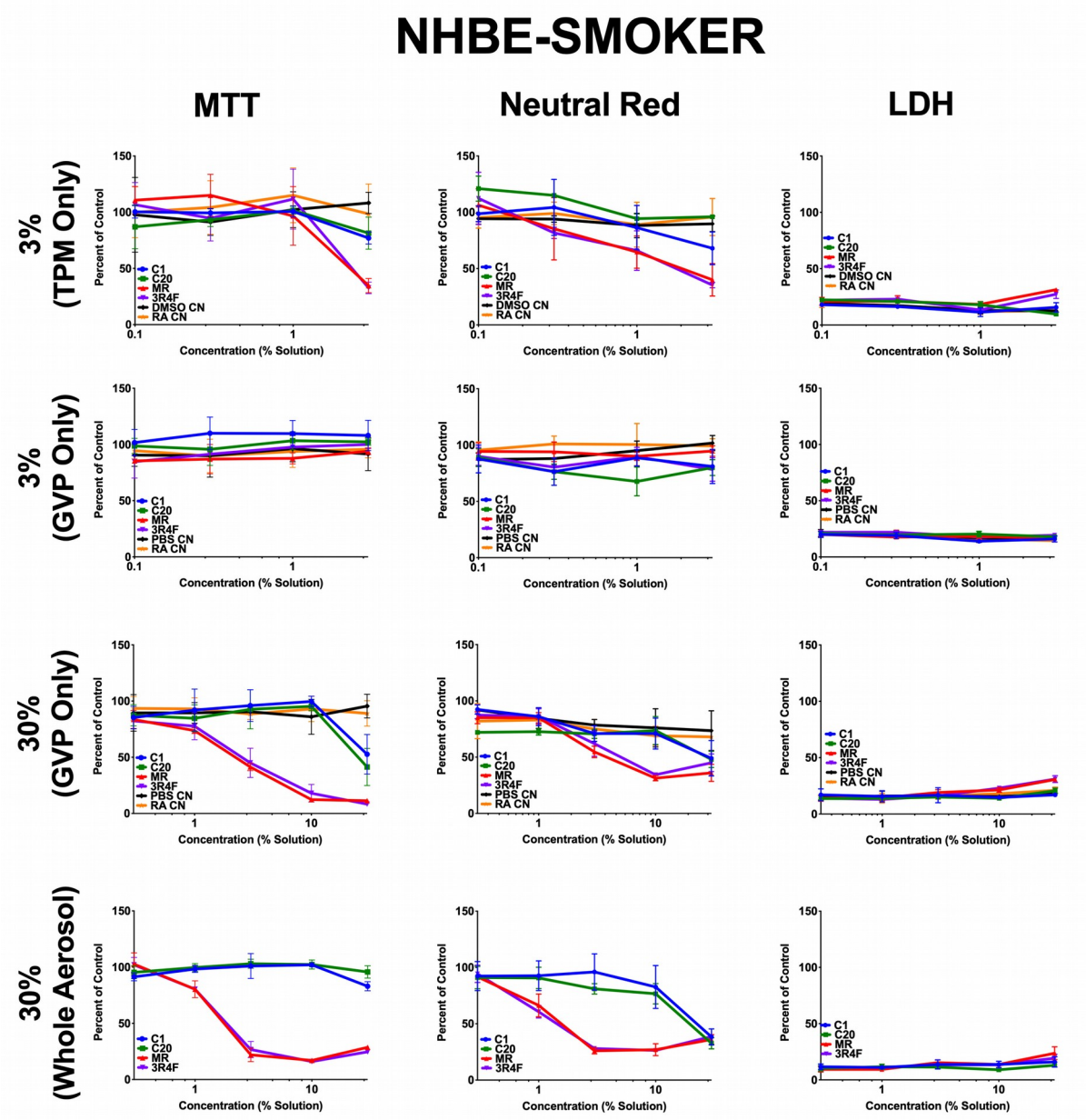
SP2. Cytotoxicity data for NIH/3T3 mouse embryonic fibroblasts. MTT, NRU, and LDH for 3% TPM, 3% GVP, 30% GVP, and 30% WA concentration- response curves. For all graphs, the x-axis = concentration in % solution, y-axis = percent of untreated control. Blue = IQOS C1, green =IQOS C20, red = Marlboro Red (MR), purple = 3R4F, black = DMSO (for TPM) or PBS (for GVP) control, orange = room air control. Each line represents the average of three experiments + SD



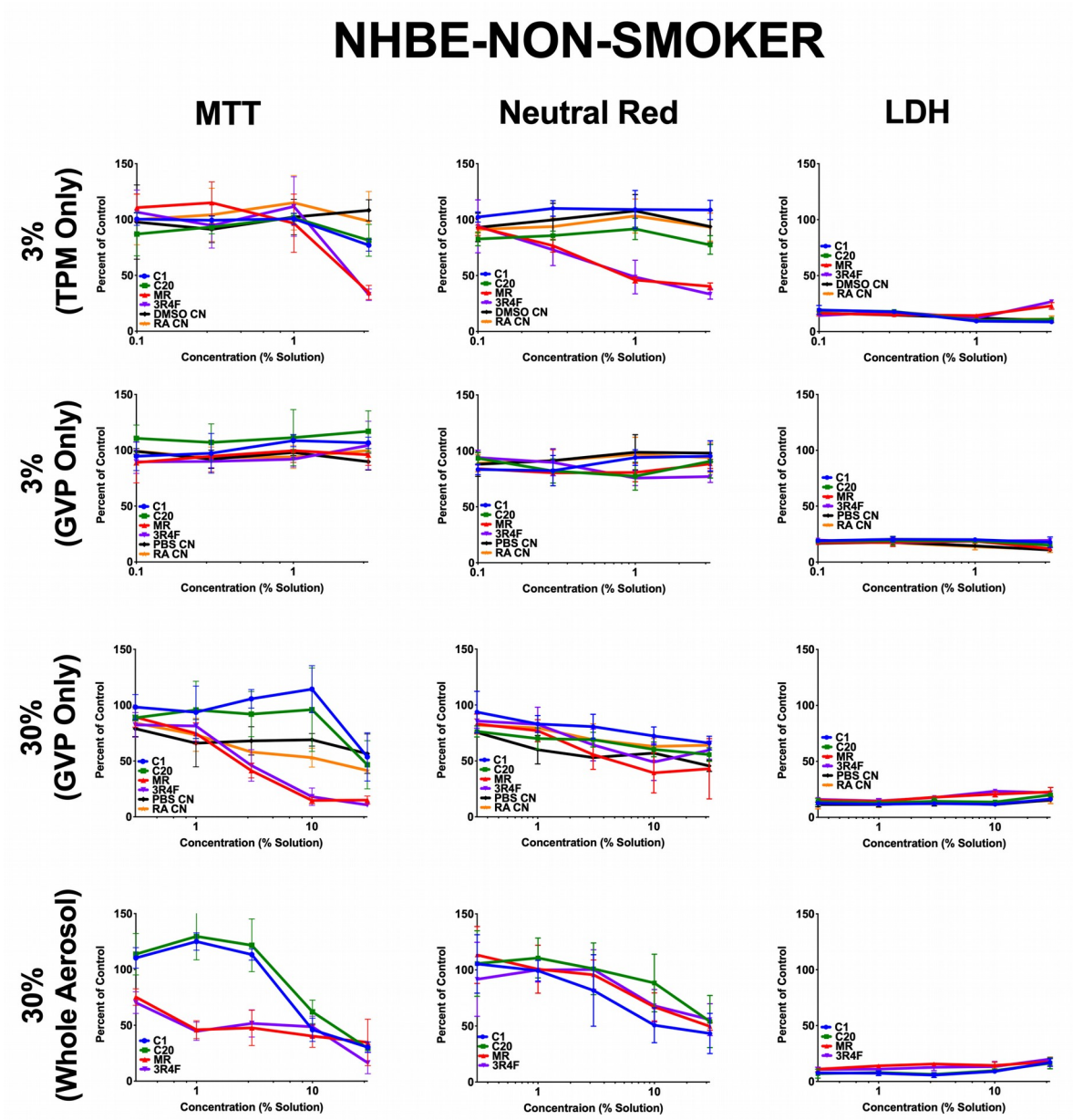
SP3. Cytotoxicity data for A-549 human lung adenocarcinoma cells. MTT, NRU, and LDH for 3% TPM, 3% GVP, 30% GVP, and 30% WA concentration-response curves. For all graphs, the x-axis = concentration in % solution, y-axis = percent of untreated control. Blue = IQOS C1, green =IQOS C20, red = Marlboro Red (MR), purple = 3R4F, black = DMSO (for TPM) or PBS (for GVP) control, orange = room air control. Each line represents the average of three experiments ± SD.



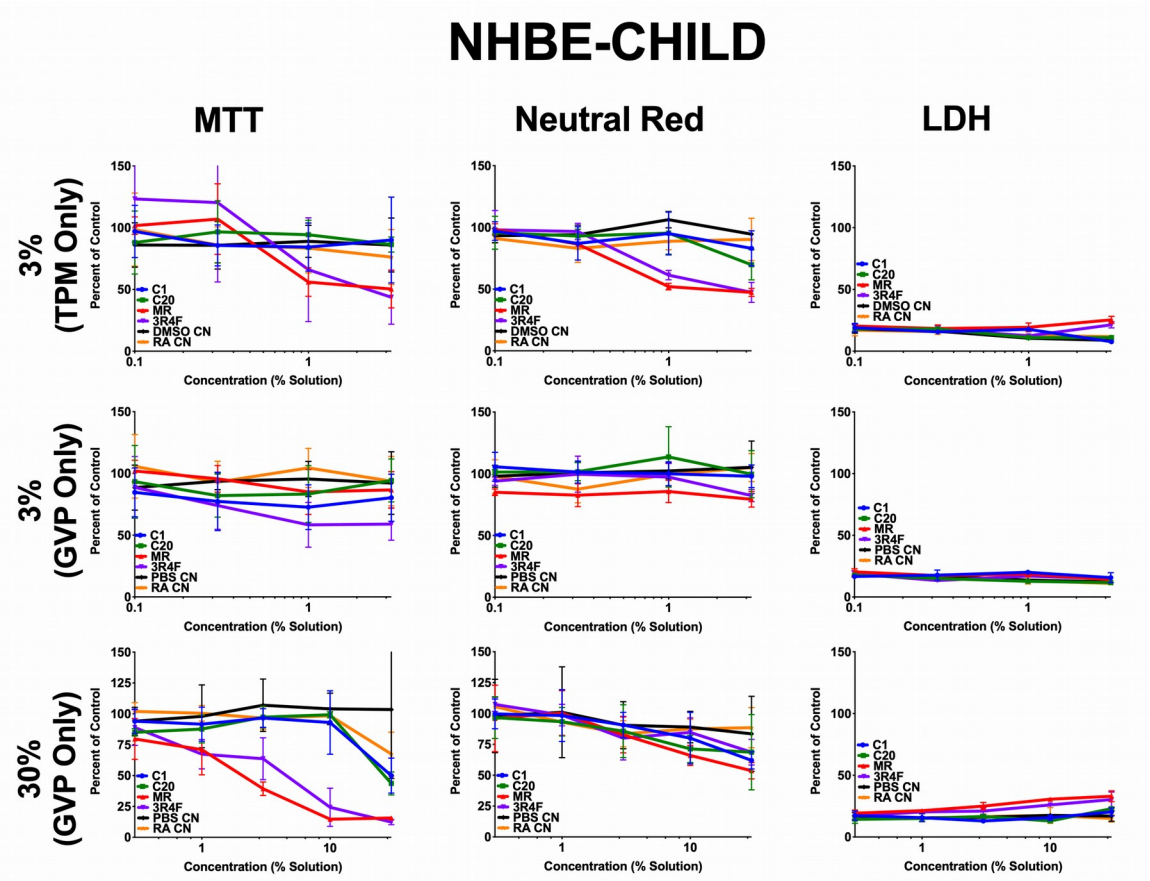
SP4. Cytotoxicity data for NHLF. MTT, NRU, and LDH for 3% TPM, 3% GVP, 30% GVP, and 30% WA concentration- response curves. For all graphs, the x-axis = concentration in % solution, y-axis = percent of untreated control. Blue = IQOS C1, green =IQOS C20, red = Marlboro Red (MR), purple = 3R4F, black = DMSO (for TPM) or PBS (for GVP) control, orange = room air control. Each line represents the average of three experiments ± SD.



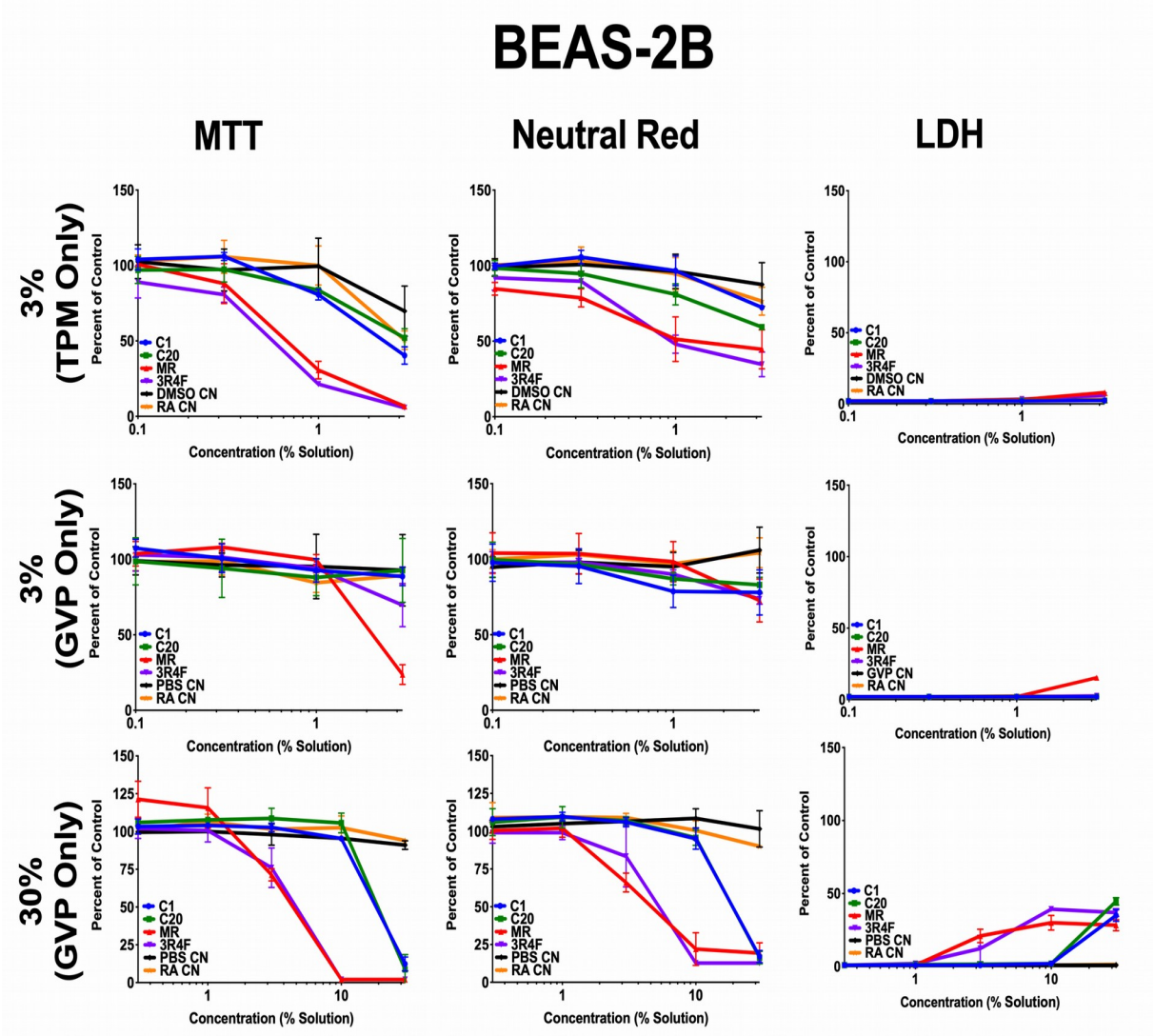
SP5. Cytotoxicity data for NHBE-Smoker. MTT, MTT, NRU, and LDH for 3% TPM, 3% GVP, 30% GVP, and 30% WA concentration- response curves. For all graphs, the x-axis = concentration in % solution, y-axis = percent of untreated control. Blue = IQOS C1, green =IQOS C20, red = Marlboro Red (MR), purple = 3R4F, black = DMSO (for TPM) or PBS (for GVP) control, orange = room air control. Each line represents the average of three experiments ± SD.



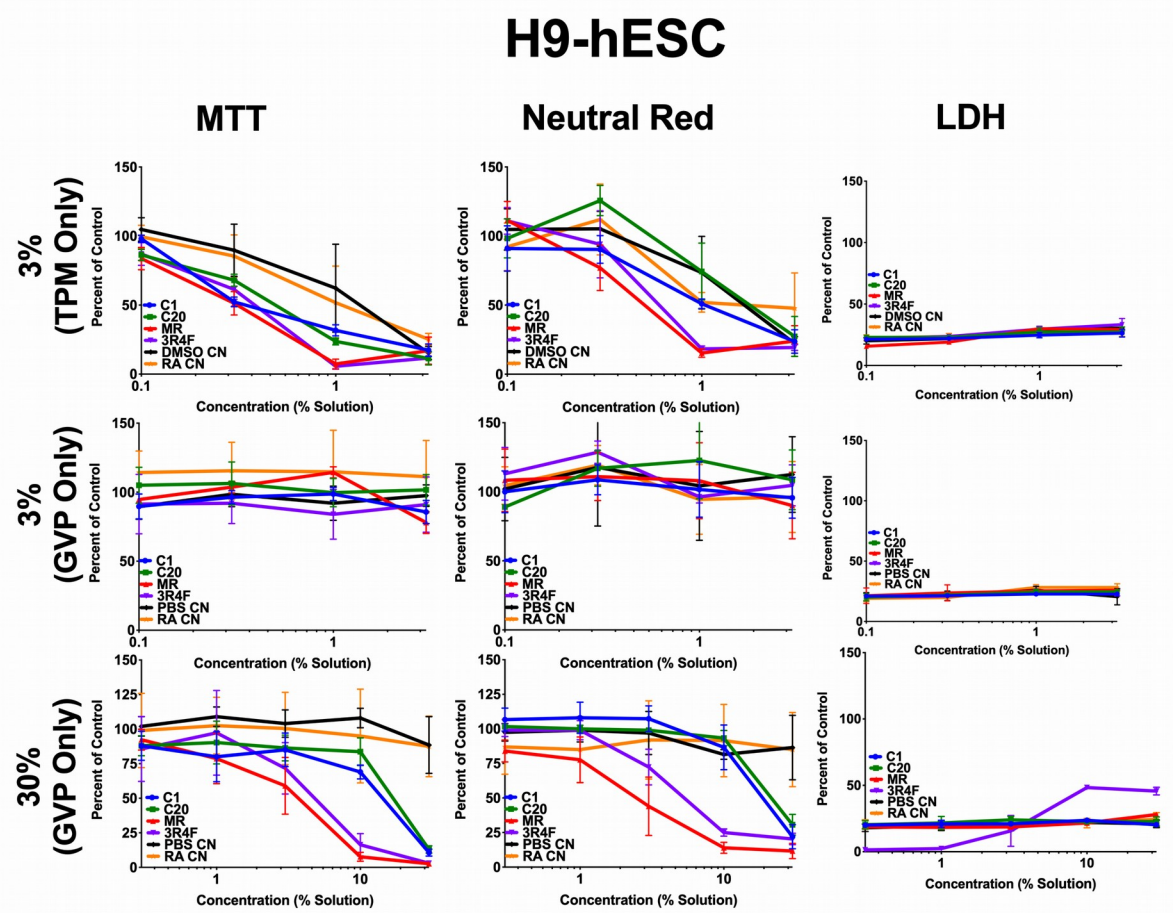
SP6. Cytotoxicity data for NHBE-Non-smoker. MTT, NRU, and LDH for 3% TPM, 3% GVP, 30% GVP, and 30% WA concentration- response curves. For all graphs, the x-axis = concentration in % solution, y-axis = percent of untreated control. Blue = IQOS C1, green =IQOS C20, red = Marlboro Red (MR), purple = 3R4F, black = DMSO (for TPM) or PBS (for GVP) control, orange = room air control. Each line represents the average of



SP7. Cytotoxicity data for BEAS-2B lung epithelial cells. MTT, NRU, and LDH for 3% TPM, 3% GVP, 30% GVP, and 30% WA concentration-response curves. For all graphs, the x-axis = concentration in % solution, y-axis = percent of untreated control. Blue = IQOS C1, green =IQOS C20, red = Marlboro Red (MR), purple = 3R4F, black = DMSO (for TPM) or PBS (for GVP) control, orange = room air control. Each line represents the average of three experiments ± SD.



SP8. Cytotoxicity data for NHBE-Child. MTT, NRU, and LDH for 3% TPM, 3% GVP, 30% GVP, and 30% WA concentration- response curves. For all graphs, the x-axis = concentration in % solution, y-axis = percent of untreated control. Blue = IQOS C1, green =IQOS C20, red = Marlboro Red (MR), purple = 3R4F, black = DMSO (for TPM) or PBS (for GVP) control, orange = room air control. Each line represents the average of three



SP9. Cytotoxicity data for H9-hESC. MTT, NRU, and LDH for 3% TPM, 3% GVP, 30% GVP, and 30% WA concentration- response curves. For all graphs, the x-axis = concentration in % solution, y-axis = percent of untreated control. Blue = IQOS C1, green =IQOS C20, red = Marlboro Red (MR), purple = 3R4F, black = DMSO (for TPM) or PBS (for GVP) control, orange = room air control. Each line represents the average of three